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Increasing the production yield of recombinant protein in transgenic seeds by expanding the deposition space within the intracellular compartment

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Seeds must maintain a constant level of nitrogen in order to germinate. When recombinant proteins are produced while endogenous seed protein expression is suppressed, the production levels of the foreign proteins increase to compensate for the decreased synthesis of endogenous proteins. Thus, exchanging the production of endogenous seed proteins for that of foreign proteins is a promising approach to increase the yield of foreign recombinant proteins. Providing a space for the deposition of recombinant protein in the intracellular compartment is critical, at this would lessen any competition in this region between the endogenous seed proteins and the introduced foreign protein. The production yields of several recombinant proteins have been greatly increased by this strategy.

Keywords: molecular farming, recombinant protein, RNA interference, protein body, PSV, prolamin, glutelin, seed protein

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Plants provide a promising platform for the production of recombinant proteins in terms of scalability, safety and cost-effectiveness and thus serve as an alternative to conventional fermentation systems that use bacteria, yeast or mammalian cells.[1-3] Specifically, seeds provide an environment for high levels of recombinant protein production, as well as a stable, ample deposition site for recombinant proteins, which allow the proteins to avoid degradation even when stored at ambient temperatures for several years.[4-6] Seed storage proteins are stored in cereal seeds at a level of 7–15% of dry seed weight, whereas dicotyledonous seeds accumulate these proteins at a level of 20–40% of dry seed weight. Prolamins are the major seed storage proteins in all cereals except rice and oat, and genes encoding prolamins are specifically expressed in the endosperm tissue.[7] On the other hand, in dicotyledonous plants such as soybean, pea and sunflower, 7S and 11S globulins are specifically and predominantly expressed in the embryos of maturing seeds.[8] Notably, the endosperm tissue of cereal seeds occupies more than 80% of the total seed weight and represents a stable storage organ. Many seed-specific promoters have been isolated from several crops and used for the expression of recombinant proteins in transgenic plants. In particular, the promoters of genes encoding major rice storage protein glutelins, such as *GluA2(gt1), GluB1* and *GluB4*, along with the strong bean phaseolin 7S and soybean glycinin 11S promoters, have been utilized for the production of several recombinant proteins, such as pharmaceuticals, in the endosperm and embryos of monocot and dicot seeds, respectively.[5,9] Seed specific expression of seed storage proteins (SSPs) is determined by the combinatory interaction between several *cis*-regulatory elements, and transcription factors recognizing the individual cognate *cis*-element have been also characterized.[10,11]

The accumulation levels of foreign recombinant proteins fundamentally depends on the yield potentiality of the target tissues (cells) of the host crop.[1,12] Furthermore, the yield of recombinant protein products is determined at both the transcriptional and post-transcriptional level. The stability of mRNA, translational efficiency and codon-optimisation of recombinant genes based on codon bias in expressed host tissue have to be taken

into consideration when designing a seed-based protein production platform.[13-15] Furthermore, the accumulation levels of recombinant protein are determined by several steps or processes leading to the final destination site of the recombinant protein after translation. Deposition in intracellular regions has a critical effect on the accumulation of recombinant SSPs that are synthesized on rough ER and are stably stocked in the specialized storage organelle referred to as the protein body (PB). To increase the accumulation level of a recombinant protein, a suitable intracellular compartment must be chosen as the deposition site. In many seeds, PBs are the main deposition sites of SSPs, which serve as nitrogen reserves for the germinating embryo. There are two types of protein bodies in rice endosperm cells, i.e., ER-derived PBs and protein storage vacuoles (PSVs).[16-18] Most dicot seed proteins are deposited into PSVs, whereas seed proteins of monocot plants are sequestered into ER-derived PBs and PSVs. ER-derived PBs contain prolamins, while 7S and 11S globulins and rice glutelin are deposited into PSVs. Although both PSV proteins and PB proteins are synthesized as secretory proteins on the rough ER, PSV proteins are transported to the PSV via Golgi apparatus or deposited directly as PAC vesicles, whereas PB proteins aggregate to form PBs in the ER lumen.[9]

The reduction of SSPs is compensated for by the increased production of other SSPs irrespective of any types of SSPs. In particular, when seeds are deficient in PSV proteins such as glutelin and globulin, the production of sulfur-poor 13 kD prolamins is preferentially increased in rice.[19] Such preferential compensation may depend on the sulfur levels of the seed proteins.[20] Therefore, when the production of some SSPs is depressed using a knock-down approach (RNA interference and anti-sense RNA) or by mutation, the production of other types of endogenous seed proteins increases due to the compensatory or rebalancing mechanisms inherent in seeds, which maintain constant levels of total seed protein. This finding indicates that seed proteins may be replaced by foreign recombinant proteins, thereby maintaining proper nitrogen levels in the seed. That is, rebalancing the proteome occurs via the regulatory mechanism required for maintaining the total seed protein content. In the seeds of low glutelin rice mutants such as LGC-1 or the a123 three glutelins-less mutant, the prolamin levels significantly increase in a compensatory manner.[21,22] The suppression of 7S β-conglycinin production in soybean seeds results in an increase in 11S glycine content via a rebalancing of seed protein content.[23] Furthermore, in transgenic rice, the suppression of genes encoding individual SSPs or a combination of several SSPs such as glutelins, prolamins and globulin significantly affects the expression of other seed protein genes, as well as PB formation, as rebalancing the proteome occurs to maintain the protein content of rice seeds.[19] Such a compensatory effect is regulated at both the transcriptional and translational levels. A similar situation was also reported in maize grains. The reduced production of both the 19 and 22 kD major α-zeins results in higher non-zein protein content.[24] These studies suggest that the production yield of foreign proteins may be effectively increased by redirecting intrinsic seed protein production to foreign protein production. The production yields of foreign recombinant proteins are greatly increased by the simultaneous reduction of the expression of endogenous seed protein genes by RNA interference (anti-sense technology) or mutation. These techniques have enabled greater amounts of *Phaseolus vulgaris* arcelin (arc) 5-1, Green Fluorescence Protein (GFP) and human growth hormone to accumulate in transgenic *Arabidopsis*, soybean and rice seeds.[25-27] Specifically, arc 5-1 levels increase to more than 24% of the total seed protein content when the amount of endogenous 2S albumins in the Arabidopsis seeds is reduced.[25] Moreover, when the soybean SSP 11S glycinin (*A1aB1b*) gene under the control of the endosperm-specific glutelin *GluB-1* promoter is introduced into low glutelin mutants such as LGC-1 or a123 lines, the accumulation levels increase approximately 2-fold compared with wild-type rice transformed with the same construct.[27] It is important to note that soybean glycinin is deposited into the same PSVs (PB-II) as the rice glutelins in transgenic rice seeds. Furthermore, the production yields of recombinant proteins are also enriched by double suppression of 13 kD prolamin (PB protein) and glutelin (PSV protein) in transgenic rice seeds.[28-30] To improve the production of human IL-10 (hIL-10), codon optimized *hIL-10* was expressed under the control of an endosperm-specific promoter while numerous prolamin or glutelin genes were simultaneously suppressed by RNA interference.[31] Notably, the significant reduction in the levels of most Cys-rich 16K, 10K and 13K prolamins, and Cys-poor 13K prolamins, was accompanied by an increase (up to 3-fold) in the yield of hIL-10 in transgenic rice seeds compared with transgenic rice seeds harbouring only *hIL-10*. The hIL-10 protein accumulated at levels as high as approximately 220 μg/grain, accounting for 18% of total seed protein. On the other hand, the severe reduction in the content of most of the glutelins (GluA, GluB, GluC and GluD) did not significantly affect the hIL-10 accumulation levels.[31] This difference may be related to the deposition site of hIL-10, since hIL-10 is mainly sequestered into ER-derived PBs referred to as IL-10 granules or PB-I, whereas glutelins are located in PB-II. Therefore, the availability of space in the final destination site (deposition site) for the desired recombinant proteins may be very important for increasing production yields. The competition for deposition sites with endogenous seed protein is lessened by the reduction in seed protein content. The availability of amino acids for protein synthesis may not be a limiting factor. The availability of deposition space for recombinant proteins is more crucial than the availability of an amino acid source. Therefore, reducing the levels of SSPs at the target deposition site for a recombinant protein represents an effective new strategy for increasing the accumulation levels of recombinant proteins.

Many recombinant proteins have been expressed as secretory proteins by ligating the signal peptide and the KDEL ER retention signal to the N and C termini of the desired recombinant protein, respectively, to improve their accumulation levels. This strategy results in higher levels of recombinant protein production than can be achieved by targeting the protein to the cytoplasm without the

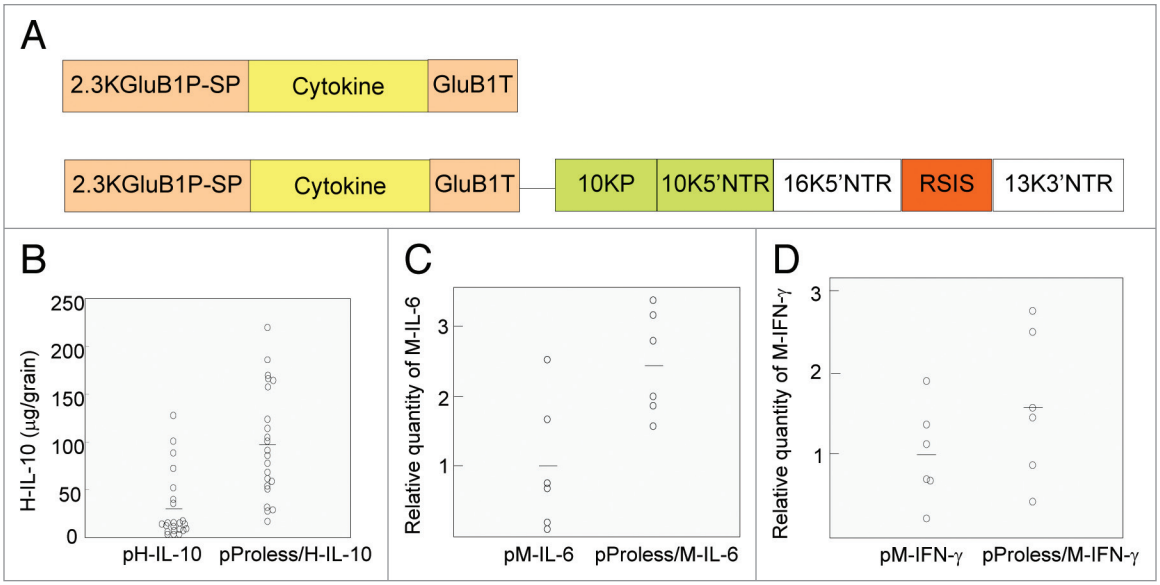

Figure 1. Increasing cytokine production by suppressing prolamin protein deposition in ER-derived PBs. (**A**) Expression constructs used for the production of cytokines, human IL-10 (H-IL-10), mouse IL-6 (M-IL-6) and mouse interferon-γ (M-IFN-γ), in transgenic rice seeds. Rice 16 kD, 13 kD and 10 kD prolamins were simultaneously suppressed by RNA interference using RSIS linked to the 5' NTR of 10 kD and 16 kD prolamins and the 3' NTR of 13 kD prolamin. (**B–D**) The protein accumulation levels were examined in more than six independent transgenic rice lines per construct. A minimum of four positive seeds from each line were analyzed by western blotting using commercially purchased human IL-10 or His-tagged as controls.

aid of signal peptides. This technique has been employed in the production of pharmaceutical proteins such as antibodies, antigens and cytokines. These recombinant proteins are mainly or exclusively deposited into the ER or ER-derived PBs in transgenic cereal seeds, although accumulation sometimes occurs in the PSV instead, due to an interaction between the recombinant protein and endogenous seed proteins.[32,33] When the 7Crp antigen peptide derived from the major T-cell epitopes from Japanese cedar pollen allergens and other types of cytokines, such as IL-4 and IFN-r, were expressed in conjunction with the simultaneous suppression of prolamin genes deposited in ER-derived PB-I,[34] these recombinant proteins were produced at high levels, as was also observed with the production of hIL-10, confirming the efficacy of this technique as a general strategy for increasing the production of recombinant proteins (**Fig. 1**). On the other hand, some recombinant proteins are trafficked and stably accumulated in PSVs based on their structural characteristics. In such recombinant proteins, a reduction in the levels of endogenous PSV proteins may be required to increase their accumulation levels via a compensatory effect. Secretory IgG, which is composed of several subunits, must be trafficked to the PSV for proper assembly to occur in transgenic rice seeds.[35] Modified PSV storage proteins containing functional peptides as fusion protein are also trafficked to PSVs. When modified glutelins containing lactostatin or novokin peptide with hypo-cholesterolic and anti-hypertensive activity were introduced into the low glutelin mutant a123, higher amounts of modified glutelins accumulated in low glutelin seeds than in wild-type seeds.[36] Higher accumulation of soybean glycine and human growth hormone in transgenic rice seeds vs. wild-type can also be explained by the increase in deposition space in the PSV caused by a reduction in glutelin content, as these recombinant proteins accumulate in PSVs (PB-II).[27,29] These findings also support the notion that allocating a desired recombinant protein to a suitable deposition site is an effective way to increase accumulation levels.

Taken together, these studies suggest that the selection of a suitable intracellular compartment is a critical step for increasing the yield of recombinant proteins in seeds, since trafficking to the final deposition site requires the proper folding, assembly and post-translation modification of the protein and is related to the accumulation capacity of the seed. The specific suppression of endogenous seed proteins at the same deposition site as the desired recombinant protein provides a new strategy for increasing the accumulation levels of recombinant proteins in seeds. Further work will be required to determine whether this strategy will be applicable to other intracellular compartments.

Disclosure of Potential Conflicts of Interest

No potential conflict of interest was disclosed.

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References

1. Twyman RM, Stoger E, Schillberg S, Christou P, Fischer R. Molecular farming in plants: host systems and expression technology. Trends Biotechnol 2003; 21:570-8; PMID:14624867; http://dx.doi.org/10.1016/j.tibtech.2003.10.002.
2. Daniell H, Singh ND, Mason H, Streatfield SJ. Plant-made vaccine antigens and biopharmaceuticals. Trends Plant Sci 2009; 14:669-79; PMID:19836291; http://dx.doi.org/10.1016/j.tplants.2009.09.009.
3. Sharma AK, Sharma MK. Plants as bioreactors: Recent developments and emerging opportunities. Biotechnol Adv 2009; 27:811-32; PMID:19576278; http://dx.doi.org/10.1016/j.biotechadv.2009.06.004.
4. Stoger E, Ma JK, Fischer R, Christou P. Sowing the seeds of success: pharmaceutical proteins from plants. Curr Opin Biotechnol 2005; 16:167-73; PMID:15831382; http://dx.doi.org/10.1016/j.copbio.2005.01.005.
5. Lau OS, Sun SS. Plant seeds as bioreactors for recombinant protein production. Biotechnol Adv 2009; 27:1015-22; PMID:19460423; http://dx.doi.org/10.1016/j.biotechadv.2009.05.005.
6. Boothe J, Nykiforuk C, Shen Y, Zaplachinski S, Szarka S, Kuhlman P, et al. Seed-based expression systems for plant molecular farming. Plant Biotechnol J 2010; 8:588-606; PMID:20500681; http://dx.doi.org/10.1111/j.1467-7652.2010.00511.x.
7. Shewry PR, Halford NG. Cereal seed storage proteins: structures, properties and role in grain utilization. J Exp Bot 2002; 53:947-58; PMID:11912237; http://dx.doi.org/10.1093/jexbot/53.370.947.
8. Ibl V, Stoger E. The formation, function and fate of protein storage compartments in seeds. Protoplasma 2012; 249:379-92; PMID:21614590; http://dx.doi.org/10.1007/s00709-011-0288-z.
9. Qu LQ, Takaiwa F. Evaluation of tissue specificity and expression strength of rice seed component gene promoters in transgenic rice. Plant Biotechnol J 2004; 2:113-25; PMID:17147604; http://dx.doi.org/10.1111/j.1467-7652.2004.00055.x.
10. Kawakatsu T, Takaiwa F. Cereal seed storage protein synthesis: fundamental processes for recombinant protein production in cereal grains. Plant Biotechnol J 2010; 8:939-53; PMID:20731787; http://dx.doi.org/10.1111/j.1467-7652.2010.00559.x.
11. Agarwal P, Kapoor S, Tyagi AK. Transcription factors regulating the progression of monocot and dicot seed development. Bioessays 2011; 33:189-202; PMID:21319185; http://dx.doi.org/10.1002/bies.201000107.
12. Schillberg S, Twyman RM, Fischer R. Opportunities for recombinant antigen and antibody expression in transgenic plants--technology assessment. Vaccine 2005; 23:1764-9; PMID:15734038; http://dx.doi.org/10.1016/j.vaccine.2004.11.002.
13. Streatfield SJ. Approaches to achieve high-level heterologous protein production in plants. Plant Biotechnol J 2007; 5:2-15; PMID:17207252; http://dx.doi.org/10.1111/j.1467-7652.2006.00216.x.
14. Takaiwa F. Transgenic rice seed as a nutriceutical delivery system. CAB Rev 2007; 2:41.
15. Desai PN, Shrivastava N, Padh H. Production of heterologous proteins in plants: strategies for optimal expression. Biotechnol Adv 2010; 28:427-35; PMID:20152894; http://dx.doi.org/10.1016/j.biotechadv.2010.01.005.
16. Benchabane M, Goulet C, Rivard D, Faye L, Gomord V, Michaud D. Preventing unintended proteolysis in plant protein biofactories. Plant Biotechnol J 2008; 6:633-48; PMID:18452504; http://dx.doi.org/10.1111/j.1467-7652.2008.00344.x.
17. Doran PM. Foreign protein degradation and instability in plants and plant tissue cultures. Trends Biotechnol 2006; 24:426-32; PMID:16843560; http://dx.doi.org/10.1016/j.tibtech.2006.06.012.
18. Krishnan HB, Franceschi VR, Okita TW. Immunochemical studies on the role of the Golgi complex in protein-body formation in rice seeds. Planta 1986; 169:471-80; http://dx.doi.org/10.1007/BF00392095.
19. Kawakatsu T, Hirose S, Yasuda H, Takaiwa F. Reducing rice seed storage protein accumulation leads to changes in nutrient quality and storage organelle formation. Plant Physiol 2010; 154:1842-54; PMID:20940349; http://dx.doi.org/10.1104/pp.110.164343.
20. Tabe L, Hagan N, Higgins TJ. Plasticity of seed protein composition in response to nitrogen and sulfur availability. Curr Opin Plant Biol 2002; 5:212-7; PMID:11960738; http://dx.doi.org/10.1016/S1369-5266(02)00252-2.
21. Iida S, Amano E, Nishio T. A rice (*Oryza sativa* L.) mutant having a low content of glutelin and high content of prolamine. Theor Appl Genet 1993; 87:374-8; http://dx.doi.org/10.1007/BF01184926.
22. Iida S, Kusaba M, Nishio T. Mutants lacking glutelin subunits in rice: mapping and combination of mutated glutelin. Theor Appl Genet 1997; 94:177-83; http://dx.doi.org/10.1007/s001220050397.
23. Mori T, Maruyama N, Nishizawa K, Higasa T, Yagasaki K, Ishimoto M, et al. The composition of newly synthesized proteins in the endoplasmic reticulum determines the transport pathways of soybean seed storage proteins. Plant J 2004; 40:238-49; PMID:15447650; http://dx.doi.org/10.1111/j.1365-313X.2004.02204.x.
24. Huang S, Frizzi A, Florida CA, Kruger DE, Luethy MH. High lysine and high tryptophan transgenic maize resulting from the reduction of both 19- and 22-kD α-zeins. Plant Mol Biol 2006; 61:525-35; PMID:16830184; http://dx.doi.org/10.1007/s11103-006-0027-6.
25. Goossens A, Van Montagu M, Angenon G. Co-introduction of an antisense gene for an endogenous seed storage protein can increase expression of a transgene in Arabidopsis thaliana seeds. FEBS Lett 1999; 456:160-4; PMID:10452550; http://dx.doi.org/10.1016/S0014-5793(99)00943-6.
26. Schmidt MA, Herman EM. Proteome rebalancing in soybean seeds can be exploited to enhance foreign protein accumulation. Plant Biotechnol J 2008; 6:832-42; PMID:18694455; http://dx.doi.org/10.1111/j.1467-7652.2008.00364.x.
27. Tada Y, Utsumi S, Takaiwa F. Foreign gene products can be enhanced by introduction into low storage protein mutants. Plant Biotechnol J 2003; 1:411-22; PMID:17134400; http://dx.doi.org/10.1046/j.1467-7652.2003.00038.x.
28. Kuroda M, Kimizu M, Mikami C. A simple set of plasmids for the production of transgenic plants. Biosci Biotechnol Biochem 2010; 74:2348-51; PMID:21071849; http://dx.doi.org/10.1271/bbb.100465.
29. Shigemitsu T, Ozaki S, Saito Y, Kuroda M, Morita S, Satoh S, et al. Production of human growth hormone in transgenic rice seeds: co-introduction of RNA interference cassette for suppressing the gene expression of endogenous storage proteins. Plant Cell Rep 2012; 31:539-49; PMID:22108719; http://dx.doi.org/10.1007/s00299-011-1191-y.
30. Yuki Y, Mejima M, Kurokawa S, Hiroiwa T, Kong IG, Kuroda M, et al. RNAi suppression of rice endogenous storage proteins enhances the production of rice-based Botulinum neutrotoxin type A vaccine. Vaccine 2012; 30:4160-6; PMID:22554467; http://dx.doi.org/10.1016/j.vaccine.2012.04.064.
31. Yang L, Hirose S, Takahashi H, Kawakatsu T, Takaiwa F. Recombinant protein yield in rice seed is enhanced by specific suppression of endogenous seed proteins at the same deposit site. Plant Biotechnol J 2012; 10:1035-45; PMID:22882653; http://dx.doi.org/10.1111/j.1467-7652.2012.00731.x.
32. Arcalis E, Marcel S, Altmann F, Kolarich D, Drakakaki G, Fischer R, et al. Unexpected deposition patterns of recombinant proteins in post-endoplasmic reticulum compartments of wheat endosperm. Plant Physiol 2004; 136:3457-66; PMID:15489278; http://dx.doi.org/10.1104/pp.104.050153.
33. Drakakaki G, Marcel S, Arcalis E, Altmann F, Gonzalez-Melendi P, Fischer R, et al. The intracellular fate of a recombinant protein is tissue dependent. Plant Physiol 2006; 141:578-86; PMID:16632592; http://dx.doi.org/10.1104/pp.106.076661.
34. Kawakatsu T, Takaiwa F. Reduction of 13 kD prolamins increase recombinant protein yield and recovery rate in rice endosperm. Plant Signal Behav 2012; 7:1402-3; http://dx.doi.org/10.4161/psb.21924.
35. Nicholson L, Gonzalez-Melendi P, van Dolleweerd C, Tuck H, Perrin Y, Ma JK, et al. A recombinant multimeric immunoglobulin expressed in rice shows assembly-dependent subcellular localization in endosperm cells. Plant Biotechnol J 2005; 3:115-27; PMID:17168904; http://dx.doi.org/10.1111/j.1467-7652.2004.00106.x.
36. Wakasa Y, Yasuda H, Takaiwa F. High accumulation of bioactive peptide in transgenic rice seeds by expression of introduced multiple genes. Plant Biotechnol J 2006; 4:499-510; PMID:17309726.